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14. ABSTRACT

The hypothesis underlying this Idea Grant project was that if it were possible to isolate and genomically analyze hundreds of single cells from prostate cancer (PCa) biopsies, that it would then be possible to create an objective diagnostic and prognostic molecular measure of primary prostate cancer that provide new insight into treating the disease. The first goal was to develop a protocol for isolating single cells from fresh biopsies that was consistent with, and not disruptive to standard histopathology procedures. We achieved this goal within the first 8 months of the project. We then proceeded to analyze 10 cases ranging from benign and high grade pre-invasive prostate cancers to intermediate level cancers scored as Gleason 6 and 7 by histopathology. We analyzed a total of over 3600 single cells from multiple biopsies of these ten cases. Our conclusions are first, that molecular measures of cancer development measured by the establishment of clonal lineages of genomically rearranged cancer cells in the prostate, as measured by copy number variation (CNV) profiles can be used to identify the earliest stages of invasive cancer; and second, that the degree of clonal development measured by CNV is directly correlated with Gleason Score. These results lead us to believe that this line of investigation should be extended to deeper DNA sequencing on a clinically relevant number of cases in order to establish prognostic molecular biomarkers for PCa.

15. SUBJECT TERMS
Prostate cancer, prognosis, diagnosis, CNV, genomics, DNA sequence, biopsy

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1. INTRODUCTION

Prostate cancer is characterized by a combination of gene mutations and genomic rearrangements that can be observed as copy number variations or CNV. The goal of the project is to examine prostate cancer biopsies from a series of individuals being treated by our co-PI and collaborator, Dr. Herbert Lepor at the Langone Center of NYU and set up a protocol that could be used in a trial to test whether cellular analysis could form the basis of an improved prognostic tool. This first objective is to develop a method for collecting individual cells from core biopsies in a fashion that preserves them in a manner suitable for genomic CNV profiling. The second objective is to begin the actual analysis. As described in the body of this report, Objective 1 has been accomplished and we have begun our work on Objective 2.

2. KEYWORDS

Prostate; cancer; biopsy; DNA copy number; DNA sequencing; biomarkers; lineage; single-cell DNA; Gleason Score

3. OVERALL PROJECT SUMMARY

We based this proposal on our hypothesis that markers for risk stratification could be optimized if molecular methods, including single cell genomic profiling could be applied to fresh biopsy tissue while still preserving the biopsy core for standard fixation and histopathology, thus permitting a direct comparison of the information available from standard and molecular prognostics.

It is our objective to execute a pilot project that would establish an operationally feasible method for obtaining single cells or nuclei from multiple prostate biopsies in real time in order to examine the potential value of prognostic information derived from a variety of molecular techniques in combination with existing pathology. Our progress against the milestones in our proposal is shown in the annotated version of the SOW below, followed by a detailed description of each accomplishment.

Annotated SOW from proposal

Objective 1 Year 1
Exfoliating cells from biopsy cores. The goal is to develop a protocol that can be used routinely for obtaining cells from fresh biopsy cores. Testing will be done on the first cases to see how many cells are released by the 'washing' protocol. Cell number can be estimated by FACS and by the total amount of genomic DNA released from the pellet.

1.1 Months -6-0: Obtain IRB approval and write informed consent document (Lepor).

1.2 Month 1: Recruit first patient and perform first core 'washing' protocol, evaluate path report (Alexander, Lepor).

1.2 MILESTONE: **ACCOMPLISHED** Receive approval from pathologist that washed cores are satisfactory (Lepor).

1.3 Month 1: Evaluate exfoliated cell suspensions as to suitability for RNA, DNA and nuclear preparation for FACS sorting (CSHL group).

1.3 MILESTONE: **ACCOMPLISHED** Satisfactory exfoliated cell suspensions.

1.4 Months 2-5: Recruit 4 more cases, and perform 'washing' protocol, checking for approval from pathologist each time (Alexander, Lepor).

1.4 MILESTONE: **ACCOMPLISHED** Settle on Standard Operating Protocol for 'washing' and prepare document.

1.5 Months 6-12: Recruit at least 15 cases for procedure and preserve cell suspensions until evaluated (Alexander, Lepor).

MILESTONE: **MODIFIED as described below.** Cells isolated from minimum of 20 cases.

Objective 2. Begins In Year 2
Performing molecular assays on cell suspensions. This includes single nucleus sequencing surveys at low read density, followed by higher read density on selected samples or cells where lineage relationships need to be established, such as in comparisons of different multifocal sites in the same case. This objective also includes the expression assays for PTEN/PHLLP and comparison of single cell data with that from the bulk DNA preparation.

2.1 Months 1-2: Sort nuclei, amplify DNA, create sequencing libraries for cells from first 12 cores. Sequence 12-24 cells from each core sample (Alexander, Hicks).

MILESTONE: **ACCOMPLISHED** Obtain satisfactory copy number profiles for >90% of sequenced cells.

2.2 Months 2-3: Perform expression assays on RNA extracted from cell suspension (Chen, Trotman)

2.3 Months 3-6: Sort and sequence 2 cores from additional cases (Alexander/Hicks)

2.4 Month 6: Evaluate methodology to ensure data quality before going on to next step. Data quality can be assessed using hierarchical clustering to determine whether clones are present, and also creating and running duplicate libraries from the same cell. (Alexander/ Hicks)

2.5 Months 7-12: Sort and sequence cores from 3 cases, Compete expression studies on bulk RNA from 5 cases. (Chen/Trotman)

2.6 Months 12-18 Sort and sequence cores from all ten cases selected in 3.1.

MILESTONE: **ACCOMPLISHED.** Ten cases complete at the survey level.

2.7 Months 14-20: Sort and sequence at high read count, samples where lineage needs to be established accurately. (Alexander, Hicks)

MILESTONE: **ACCOMPLISHED**, Complete single nucleus sequence and lineage studies on 10 cases.

WORK IN PROGRESS. Complete expression studies on ten cases

Objective 3.
Selecting cases for sequencing and clinical evaluation. This decision-making process will be performed by Dr. Lepor in consultation with the other members of the collaboration.

3.1 Months 6-12: Select 10 cases from the 20 or so cases from which cells have been collected

MILESTONE: **ACCOMPLISHED**, Find 2 benign cases, 2 with HGPIN, 2 with ASAP and 2 multiFocal and 2 with mixed Gleason scores (Lepor)

3.2 Months 12-18: Collect more cases if necessary (Lepor)

3.3 Months 12-18: Begin clinical data analysis, matching single cell lineage relationships with clinical data including Gleason scores. (Lepor/Hicks/Wigler)

MILESTONE: **MANUSCRIPT IN PREPARATION.** Publication of results

Summary of progress on Objective 1. Objective 1 was to establish a protocol for obtaining a large number of single cells from multiple prostate cancer biopsies in manner that was consistent with and non-disruptive to normal fixing procedure used to prepare biopsied tissue for histopathology. Further, we wanted the preparation method to be simple and quick enough to perform in real time in the urological operating suite. The method we tested in Objective 1 is shown in **Figure 1**.

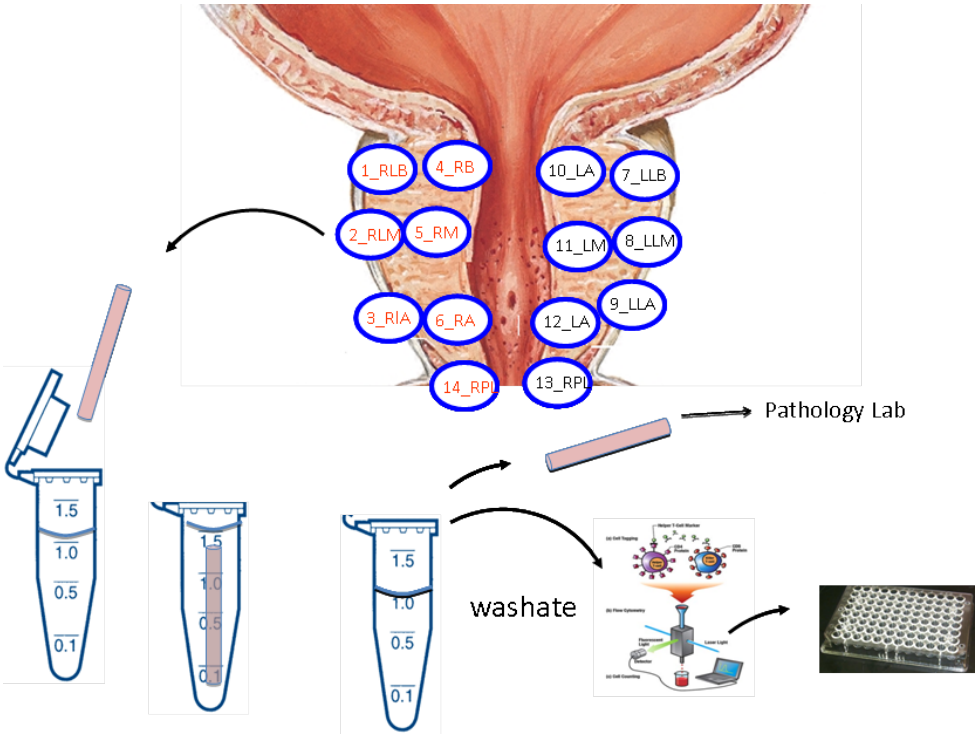
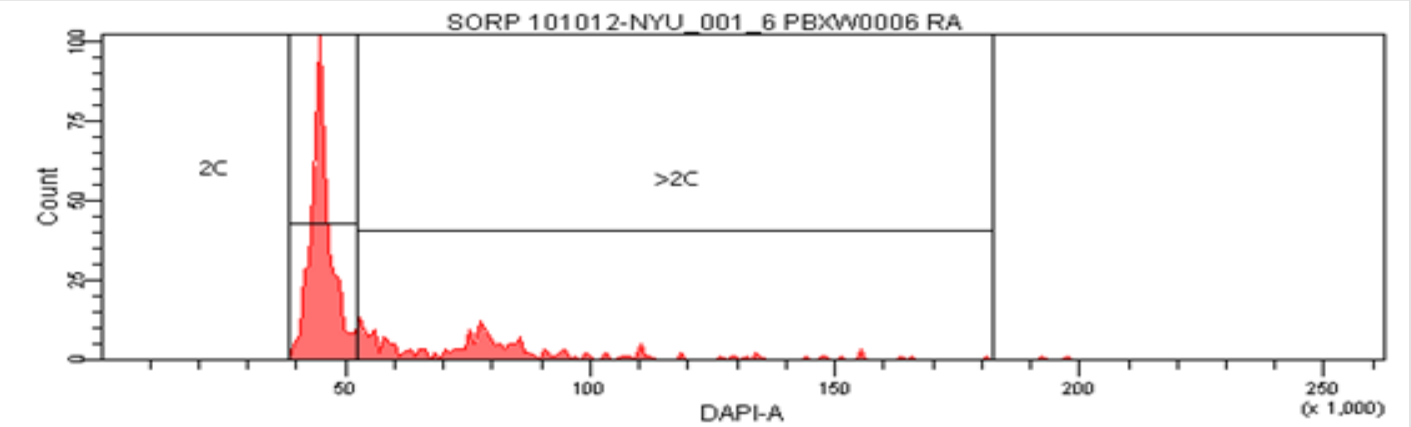


Figure 1. Schematic cell collection method from fresh biopsies performed in Dr. Lepor's urological operating suite. Biopsy tissue removed from the needle is washed briefly in PBS buffer for 30 sec. and then transferred immediately to fixative as part of normal histopathology procedure. Cell suspension is frozen until preparation for Fluorescence Activated Cell Sorting FACS, where single cells or single nuclei are distributed, one cell at a time, into 96 well plates for DNA amplification and CNV profiling.

We completed the goals of Objective 1, achieving all procedural and methodological milestones. As outlined in the SOW, the first 5 months of the project were concerned with first, obtaining essential approvals for the project and second, a series of trial runs of the biopsy washing protocol to minimize disruption of the normal pathology procedure. During the trial period, the washing protocol was modified to maximize the number of exfoliated cells collected per core needle biopsy washing while maintaining the integrity of the tissue biopsy for downstream histological evaluation. After collection, the washings were transferred to CSHL where isolated nuclei were sorted (see Figure 1) then processed for single nucleus sequencing.

Figure 2: Histogram. Isolated nuclei from a core needle biopsy washing that was scored as a Gleason 7 (4+3) by the pathologist. Gates were set at 2C (diploid) and >2C (aneuploid) with ~25-50 single nuclei collected per biopsy washing. Sorted sample is NYU001 Gleason7 (Sector 6_Right Apex).



By month 6 of year 1 we had begun to perform actual genomic copy number profiling on single cells from actual biopsies. It became clear that the profiling data, some of which is presented below, was leading us toward selectively studying more early stage patients rather than taking all patients in sequence. Thus, we altered the plans for Objective 1.5 which had been to accumulate washings from 15 patients before proceeding to single cell analysis. Instead we began a more Bayesian approach whereby we perform single cell analysis on one or two patients and choose subsequent patients based on the results of the accumulating data. Thus, by month 12 of year 1 we had performed CNV analysis of 5 cases with varying Gleason scores as described in the summary for Objective 2.

Summary of progress on Objective 2 (Year 1)

As described in our previous progress report, the first Milestone for Objective 2 was accomplished in Year 1 as we showed that the methodology for capturing and CNV profiling of cells from biopsy washes was feasible and that we could profile a complete case (14 cores) at the minimal level of 12-24 cells per biopsy.

2.1 Months 10-12: Sort nuclei, amplify DNA, create sequencing libraries for cells from first 12 cores. Sequence 12-24 cells from each core sample (Alexander, Hicks).

MILESTONE: **ACCOMPLISHED** Obtain satisfactory copy number profiles for >90% of sequenced cells.

Summary of progress on Objective 2 (Year 2)

During Year 2 we proceeded as planned in collaboration with Dr. Lepor who picked 12 cases representing various stages of prostate cancer from benign to early advanced (Gleason 7 (4+3). In the course of this work, we achieved the major milestones that we had proposed, with the exception of performing the expression profiling of the PTEN and PHLLP genes in the bulk preparations. After some consideration of our initial results we decided that the mRNA expression assays from the bulk tissue would be too contaminated with normal cells for a useful result to be obtained. We therefore plan to do these assays on single cells, however, the technology for single cell RNA is still in development and will be accomplished under separate funding. The rest of work on DNA CNV profiling is described below.

2.6 Months 12-18: Sort and sequence cores from all ten cases selected in 3.1.

MILESTONE: **ACCOMPLISHED** Ten cases complete at the survey level.

2.7 Months 14-20: Sort and sequence at high read count, samples where lineage needs to be established accurately. (Alexander, Hicks)

MILESTONE: **ACCOMPLISHED**, Complete single nucleus sequence and lineage studies on 10 cases. **MODIFIED WORK IN PROGRESS.** Complete the expression studies on ten cases for PTEN related genes.

Specific results from Objective 2 (Year 2)

As part of our collaboration with Dr. Lepor at NYU Langone Medical Center, we have collected prostate biopsy washings from 12 patients and have successfully performed single nucleus sequencing (SNS) on over 3600 cells from 122 anatomic regions of 10 of those cases, covering a broad range of histological states from benign to high grade intraepithelial neoplasia (HGPIN) to low, intermediate and advanced prostate cancer (PCa) as listed in Table 1.

Single cell genomic profiling of approximately 3600 single nuclei from 122 prostate biopsy washings has been completed. For interactive data visualization and interpretation, we have constructed a novel integrated single cell genomics viewer (SCGV). The data are displayed within the SCGV as a combined heatmap and dendrogram with menu bars of enhanced options, all featured in an intuitive and user-friendly interface. Thus, it is possible to simultaneously view hundreds of genomic profiles of the nuclei sampled from multiple regions within the prostate gland. Many features of genomic complexity, such as degree and frequency of genomic

Table 1. Case Descriptions

Case	Age	Pre-treatment PSA (ng/mL)	Sectors	Gleason Score Final*	Clonal	Subclones	Cells Total	Unstable Genomes	Cells Shredded
nyu003.benign.1	47	3.51	13	----	no	no	317	33	7
nyu009.benign.2*	56	11	14	----	NA	NA	319	NA	NA
nyu002.pin.1	72	1.6	13	----	no	no	679	70	19
nyu005.GL6.2	64	4.06	14	6 (3+3)#	yes	no	325	50	16
nyu008.GL6.3	60	4.5	2	6 (3+3)	no	no	78	10	2
nyu001.GL7.1	63	6.08	14	7 (3+4)	yes	yes	725	130	19
nyu007.GL7.2	65	10.6	13	7 (3+4)^	yes	yes	290	70	11
nyu010.GL7.3	79	7.3	15	7 (3+4)	yes	no	344	110	3
nyu004.GL7.4	75	19	14	7 (4+3)#	yes	yes	321	20	9
nyu011.GL7.5	63	9.53	10	7 (4+3)	yes	no	229	36	8
Average Age	64	Total	122	-----	-----	-----	3627	529	94

***** Gleason score of radical prostatectomy specimen: **#** Downgraded, **^**Upgraded

***** Analysis pending

instability, clonal emergence, heterogeneity among clones, ploidy violations, overlap with common patterns of CNV and clone migration/trafficking, which cannot be readily observed by standard histopathology, are easily identified by our methodology and analysis.

The SCGV heatmap is a matrix where chromosomes are ordered in rows from 1-22 followed by X and Y (on the left side of SCGV image) and the individual copy number profiles of nuclei are arranged in columns. Legends along the right side of the SCGV image describe the color-coding scheme for both copy number and selected sectors while the ploidy legend is set to a grayscale. A color bar along the bottom of the heatmap identifies the sector of origin for each nucleus and beneath it is a grayscale bar denoting ploidy for that nucleus. Within the heatmap, white denotes a genomic region that is copy number 2 while blue and red specify regions of deletion and amplification, respectively. The numeric scale along the left side of the dendrogram represents the cluster merging distance where height is relative to distance such that a node with the maximum height represents a cluster with the largest distance between its members.

Our team of computational scientists and quantitative biologists at CSHL have generated the bioinformatic tools and statistical methods needed for complex genome analysis. They continue to make improvements to our single cell genomic viewer (SCGV), a novel platform created for interactive data visualization and interpretation. The SCGV integrates anatomy and pathology with copy number profiles (CNP) and phylogeny. The data is displayed within the SCGV as a combined heatmap and dendrogram with menu bars of enhanced options. Thus, it's possible to view data from hundreds of cells to a single cell within sampled regions of a tumor. The cells are clustered by their CNP highlighting clonal differences and anatomic location within the prostate. The CNP of a single cell to multiple cells can be viewed at various levels of magnification. This visualization software is user-friendly and sufficiently flexible for upgrading as new and improved algorithms are developed. Efforts are underway to incorporate the SCGV into a web-based interface so that any clinical or statistical collaborator can access the data and its associated metrics.

Preliminary data from our exploratory study provides a detailed image of prostatic neoplasia as parameters of genomic complexity, including clonal emergence, degree and frequency of genomic instability, heterogeneity among clones, clonal dispersion and spread, ploidy and overlap with common patterns of copy number variations (CNVs) are easily identified by our methods and analysis within the SCGV. Several noteworthy features have emerged in the course of our work, foremost is the strong correlation of pathological assessment (Gleason score) with single cell genomic data. What the pathologist sees as neoplasia correlates to what we see at the single cell genome level in terms of the emergence of clones with genomic complexity, aneuploidy and instability. There is overwhelming concordance between a Gleason scored tumor of ≥ 7 and our identifying an emerging clone(s) as shown in the SCGV images in **Figures 3** through **5**.

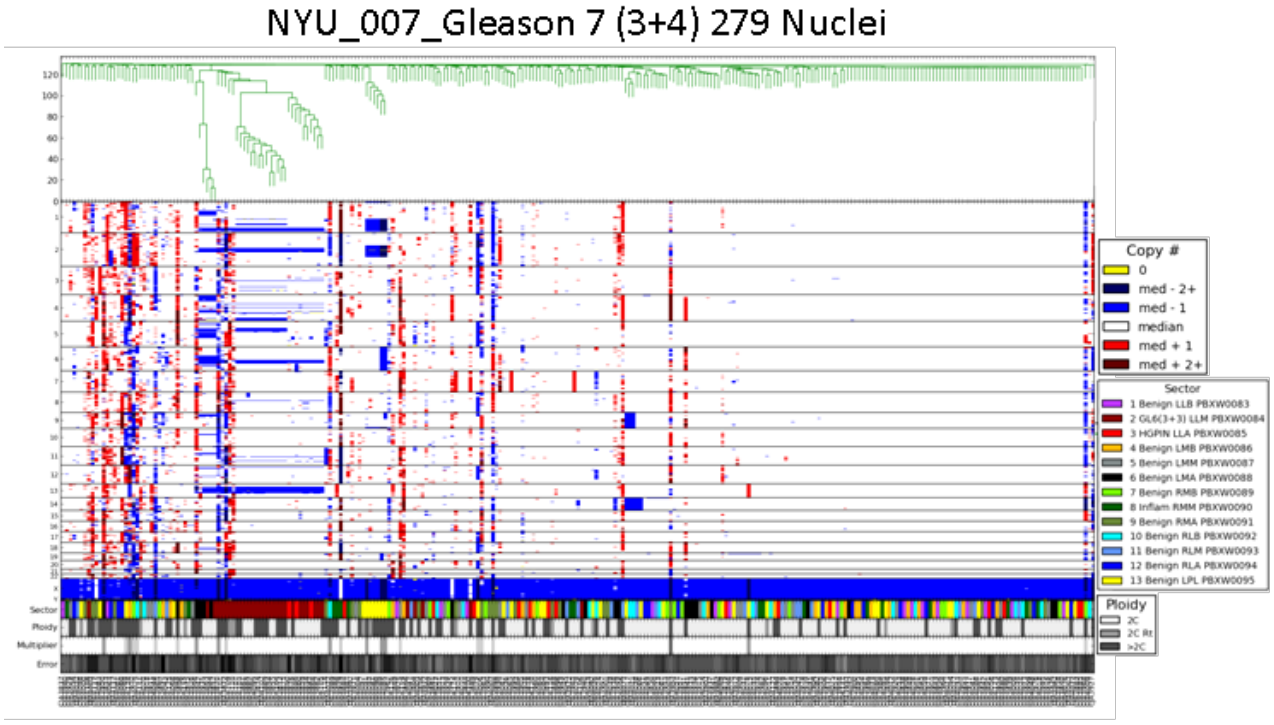


Figure 3: Single cell genomic viewer. The combined heatmap and dendrogram for case NYU007 Gleason7 (3+4) showing copy number alterations across genomes for 279 nuclei profiles from prostate biopsy washings from 13 sectors. A large clone with three diverging subclones is present and there are cells sampled from the Left Medial Apex (LMA) colored black on the sector color bar which was deemed "benign" by pathologist but clearly showing evidence of clonality. An independent clone colored yellow on the sector color bar from the Left Posterolateral (LPL) mid-gland peripheral zone which also had been called "benign" by pathologist is featured as well (as in magnified SCGV image in Figure 4).

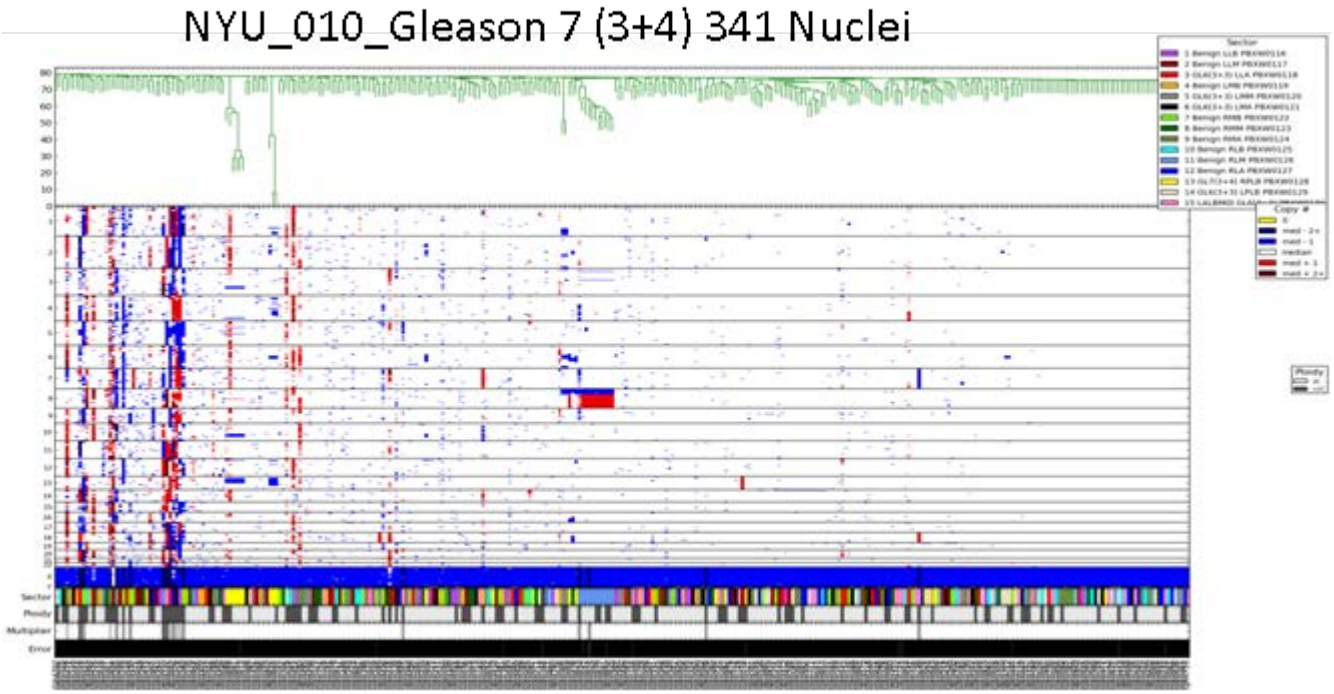


Figure 4: Single cell genomic viewer. The combined heatmap and dendrogram for case NYU010 Gleason7 (3+4) showing CNVs across genomes for 341 nuclei profiles from prostate biopsy washings from 15 sectors. Multiple small independent clones are visible.

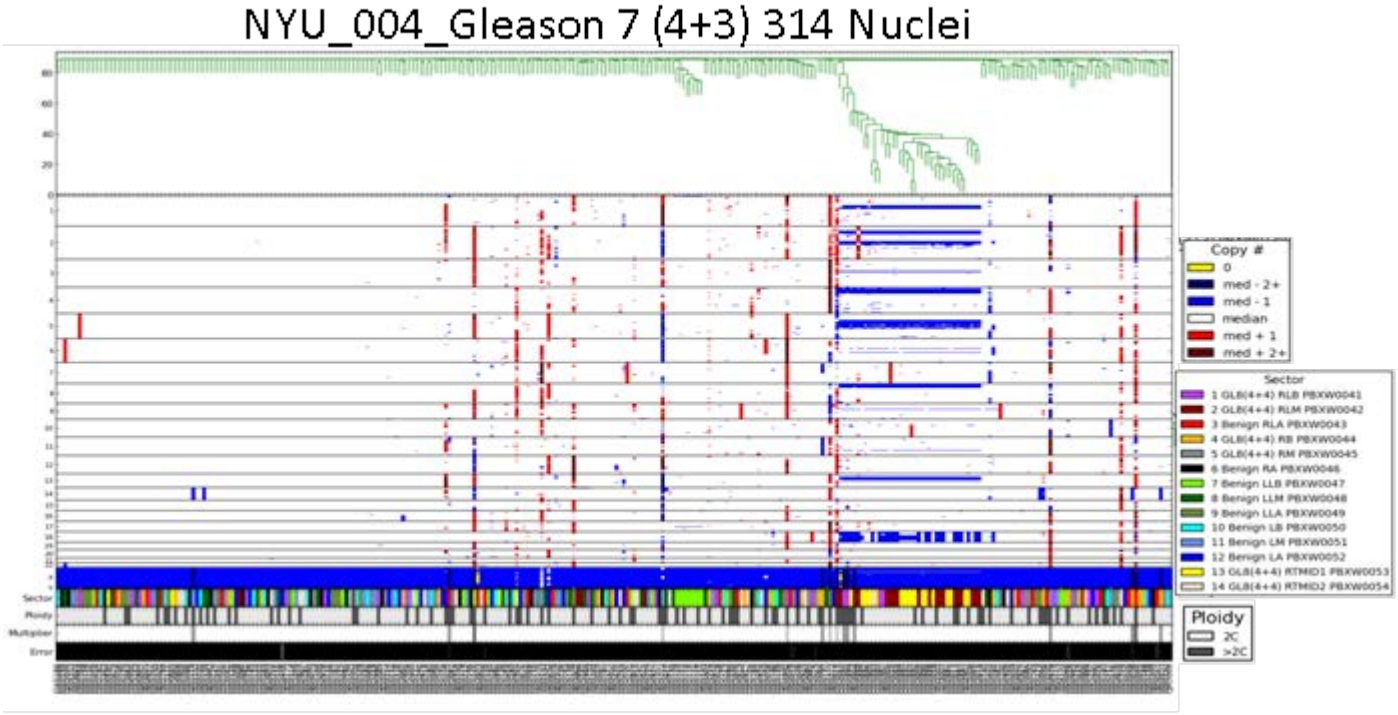


Figure 5: Single cell genomic viewer. The combined heatmap and dendrogram for case NYU004 Gleason7 (4+3) showing CNV across genomes for 314 nuclei profiles from prostate biopsy washings from 14 sectors. A large clone with several smaller subclones is present.

We have encountered instances of discordance between pathologic and genomic observations. In samples where the pathologist's call is "benign", we sometimes observe clones by genomic analysis (as in Figure 4). This is likely due to the increased sensitivity of our method over standard histopathology. However we cannot rule out the possibility of under-sampling of the paraffin blocks of prostate core biopsies. Therefore

in such instances, deeper levels will be cut which may reveal significant lesions. Likewise in the reverse instance, where the pathologist's call is "malignant" yet no clones are observed, under-sampling of individual nuclei is a possible reason. In that case, additional nuclei from the core biopsy will be sampled for genomic analysis.

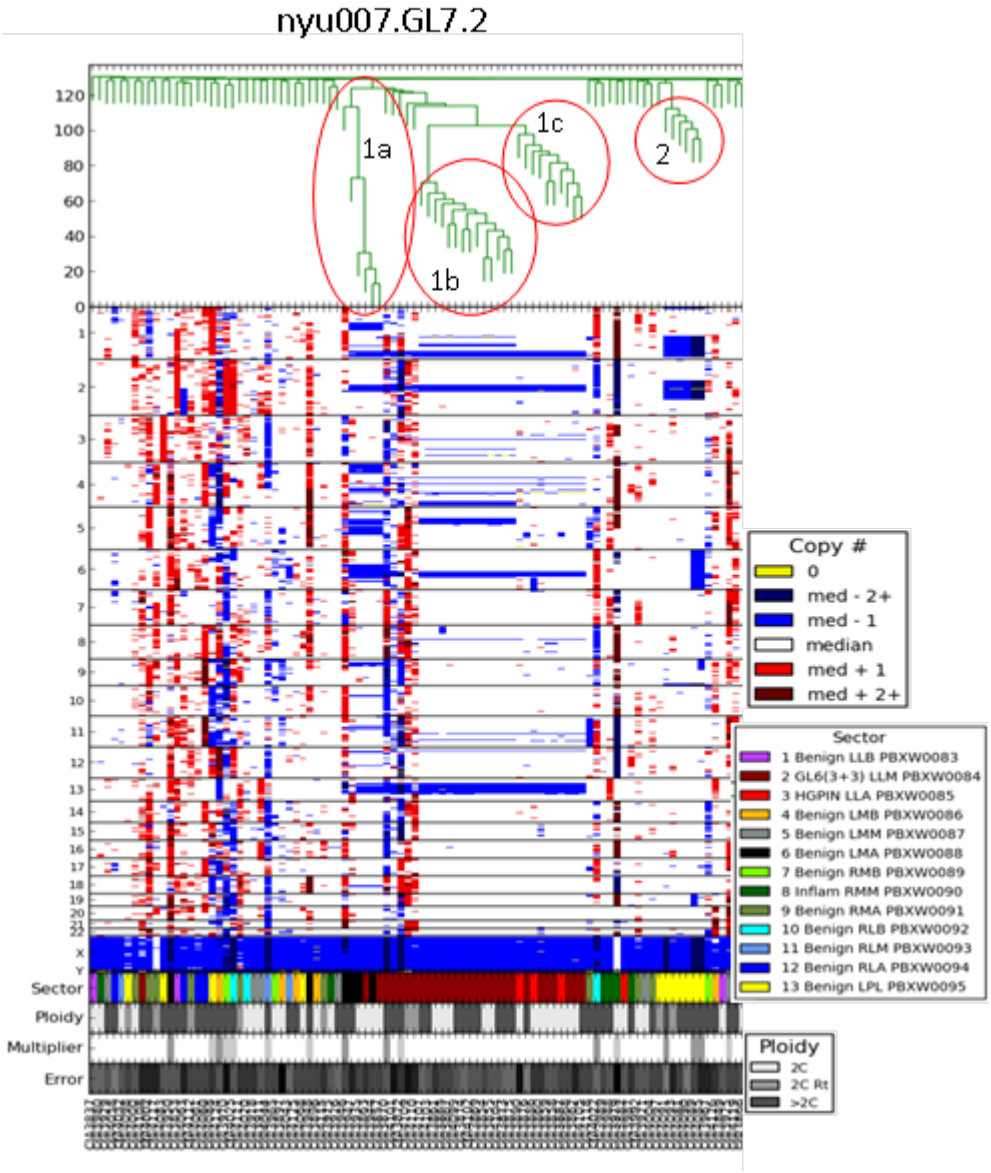


Figure 6: Single cell genomic viewer highlighting clonality. A magnified view of SCGV image of NYU007 Gleason 7 (3+4) showing a large clone with 3 divergent subclones coming from neighboring anatomic regions of the prostate including: Left Lateral Mid (LLM), Left Lateral Apex (LLA), and Left Medial Apex (LMA). Note that two of the sectors (6 LMA and 13 LPL) were called "benign" by pathologist but have cells comprising clones. The smaller independent clone (sector 13 in yellow) comprised of cells from the left posterolateral (LPL) mid-gland with large deletions on chromosomes 1q and 2p/q and additionally 2 cells have a further deletion of chromosome 6 as seen in CNP (as in Figure 7).

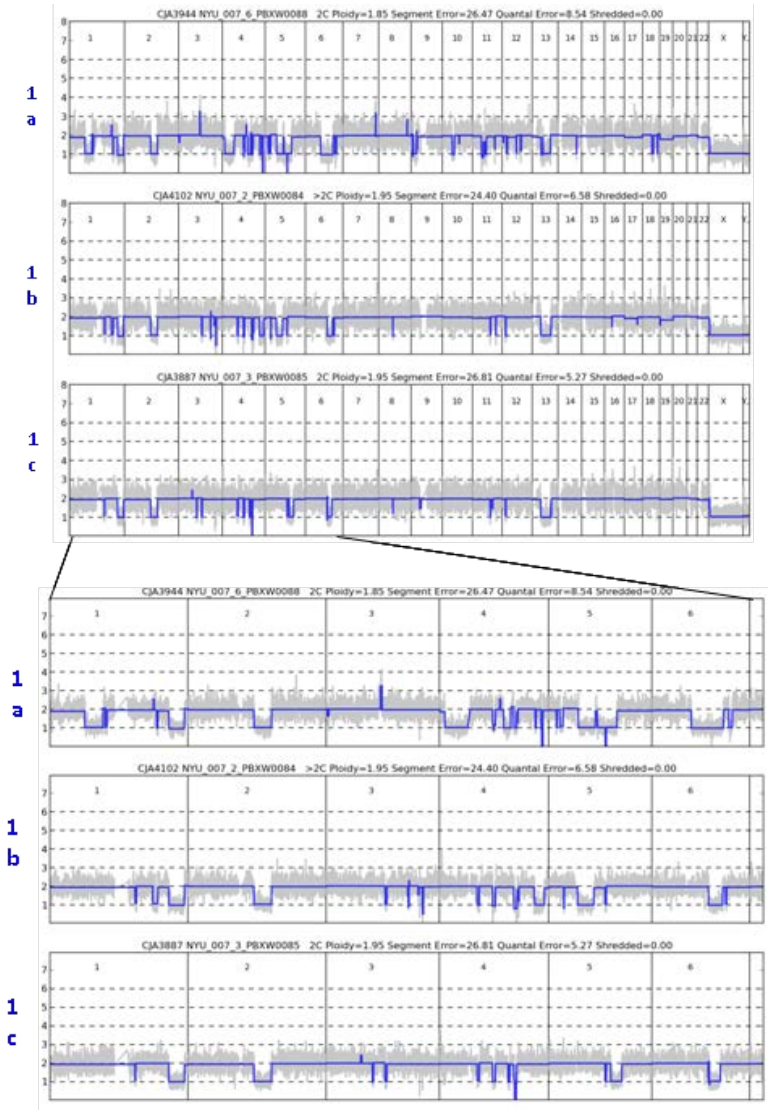


Figure 7: Genome plots of representative cells from 3 subclones of NYU007 Gleason 7 (3+4). Genome plots of normalized bin counts (gray line) and segmentation (blue line) corresponding from top to bottom to Sectors: #6 LMA (subclone1a), #2 LLM (subclone 1b) and #3 LLA (subclone 1c). These subclones contain several large deletions across the genome (panel A). A magnified view of chromosomes 1-6 (panel B).

4. KEY RESEARCH ACCOMPLISHMENTS

1. We proved that washing the biopsies will yield sufficient cells for genomic profiling and that the cells yield sufficient DNA for sequencing and CNV analysis.
2. We developed an efficient methodology for single cell analysis by DNA copy number that does not disrupt normal pathology processing or evaluation.
3. Our analysis indicates that genome complexity as measured by CNV profiling is proportional to standard pathology grading (Gleason Score).
4. From the cases we have studied it is clear that, as measured by the presence of clonal CNV lineages that prostate cancer can initiate at multiple sites in the prostate in a single individual.

REPORTABLE OUTCOMES

The Key Research Accomplishments described above will form the basis for a publication in the near future, however, there is additional mathematical and bioinformatics analysis required to put a more quantitative foundations under our initial conclusions.

5. CONCLUSIONS

We conclude from our two years' work that:
1. We have developed a feasible method for obtaining cells from prostate biopsies that does not disrupt normal histopathology protocol;
2. The copy number profiling data obtained from individual cells are reproducible;
3. As originally proposed, the copy number profiling data can be interpreted to reflect the initiation of cancer progression, using the extent of clonal organization as a metric for the degree of cancer progression;
4. On the basis of the ten samples analyzed so far, clonal structure is apparently highly correlated with Gleason Score.
5. Although it is too early to determine whether our long term goal of developing a prognostic tool will be realized, we have come a long way toward developing the technical and mathematical tools necessary for achieving that goal.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

Abstracts

CSHL Biology of Single Cells Meeting. March 2013
Tracing tumor lineage and progression through genomic copy number profiling at the single cell level.
Joan Alexander, Jude Kendall, Timour Baslan, Dan Levy, Kandasamy Ravi, Jen Troge, Jean McIndoo, Asya Stepansky, Hilary Cox, Mike Riggs, Anthony Leotta, James Hicks and Michael Wigler.
Cold Spring Harbor Laboratory, Cold Spring Harbor NY 11724

James Hicks - Presentations

AACR Annual Meeting 2014
Temporal dynamics of cancer at the single cell level
Saturday, Apr 05, 2014, 3:39 PM - 3:59 PM
James B. Hicks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

AACR Annual Meeting 2014
Single cell genomic and clonal analysis of breast and prostate tumor biopsies
Tuesday, Apr 08, 2014, 3:39 PM - 3:59 PM
James B. Hicks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

CSH Asia Biology of Single Cells Meeting
1000's of cells – 1000's of genomes
December 8-12, 2014
James B. Hicks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Invited Seminars

Oncology Grand Rounds
Monter Cancer Center, Northshore LIJ Medicial Center
Analyzing Cancer progression at the single cell level
August 16, 2013
James B. Hicks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Dept. of Statistics, Purdue University
1000's of cells – 1000's of genomes

Oct 7, 2014
James B. Hicks. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Michael Wigler - Invited Seminars

Pathology Department
SUNY Stony Brook, NY
“Analysis of Cancer One Cell at a Time”
March 20, 2014
Michael Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Symposium on Personalized Medicine
Penn State in Hershey, PA
“Applications of Single Cell Analysis in Oncology”
May 16, 2014
Michael Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Single Cell Symposium
The Karolinska Institute, Sweden
“Single-Cell Genomics”
September 9-11, 2014
Michael Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

7. INVENTIONS, PATENTS AND LICENSES

None

8. REPORTABLE OUTCOMES

The Key Research Accomplishments described above will form the basis for a publication in the near future, however, there is additional mathematical and bioinformatics analysis required to put a more quantitative foundations under our initial conclusions.

9. OTHER ACHIEVEMENTS

None

10. REFERENCES

None

11. APPENDICES

None